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The Roles of Influenza Virus Haemagglutinin and Nucleoprotein in Protection: Analysis Using Vaccinia Virus Recombinants

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Vaccinia virus recombinants expressing haemagglutinin (HA) or nucleoprotein (NP) from influenza virus A/PR/8/34 were used to investigate protective immunity in mice, with two protocols. Protection was assessed by mortality and morbidity rates and by lung virus titres after infection intranasally with A/PR/8/34. In the first protocol, mice immunized with vaccinia-HA recombinant virus and infected intranasally with A/PR/8/34 were almost totally protected, but mice immunized with vaccinia-NP virus were very poorly protected. In the second protocol, the recombinant viruses were used to stimulate in vitro T cells that are specific for HA and NP; both populations of T cells, when transferred to A/PR/8/34-infected mice, afforded good protection. The results indicate that an immune response specific for just HA provided protection that was almost indistinguishable from that provided by whole A/PR/8/34. On the other hand, immunization with vaccinia-NP provided poor protective immunity, despite the fact that transferred NP-specific T cells were very effective and vaccinia-NP immunization has previously been shown to stimulate cytotoxic T cells. These results demonstrate that a single viral antigen, delivered by live vaccinia virus, can provide effective protection, but that immunization for cross-protection against heterologous influenza virus remains elusive.

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Effective vaccines against influenza virus have been difficult to develop because the antigenicity of the virus changes with time [7]. Influenza virus-specific antibody is not cross-reactive on different influenza virus subtypes and protects mice against re-infection with homologous virus but offers little protection against heterologous virus [13, 14]. A subset of cytotoxic T cells (Tc), on the other hand, is cross-reactive; that is, it recognizes all influenza A virus subtypes [4]. Transferred cross-reactive Tc protect mice against infection with homologous or heterologous virus [9, 15]. This protection is manifested as a reduction in lung virus titre and protection against mortality.

Several different vaccinia recombinant viruses, each expressing a single foreign viral antigen, have been used to protect experimental animals from a variety of viral diseases [5, 11, 15, 16]. In this paper vaccinia recombinant viruses expressing either haemagglutinin (HA) or nucleoprotein (NP) from influenza virus are used to examine the role of those two antigens in the protection of mice against lethal infection with influenza virus. Published work shows that HA stimulates virus-neutralizing antibodies and subtype-specific Tc, while NP stimulates non-neutralizing antibody and cross-reactive Tc [1, 2, 12, 19]. This paper investigates whether a response to HA alone is sufficient to protect mice and whether the NP-specific Tc primed in mice immunized with vaccinia-NP recombinant

virus [1, 19] have any role in protection. Protection was assessed as reduction in mortality, morbidity, and lung virus titres.

MATERIALS

Animals Specific pathogen-free mice were bred at the John Curtin School of Medical Research and used at 6–7 weeks of age.

Viruses. Influenza virus A/PR/8/34(H1N1) was grown in the allantoic cavity of 10-day-old embryonated eggs. The infectious allantoic fluid was aliquoted and stored at −70°C. The construction of the vaccinia recombinant viruses VV-PR8-HA6 and VV-PR8-NP6, expressing the HA and NP respectively from A/PR/8/34, has been described previously [1]. A thymidine kinase negative vaccinia virus WR strain, VV-WR-TK⁻, was generously provided by Dr B. Moss (NIH, Bethesda, Md. USA).

Assay for protection of immunized mice. Mice were immunized intravenously (i.v.) with either 10^7 plaque-forming unit (p.f.u.)-vaccinia virus or 400 HAU influenza virus. After 21 days the mice were challenged with between 10 and 1000 LD_{50} influenza virus administered intranasally (i.n.) after light ether anaesthesia. Mouse mortality and morbidity were monitored daily for a further 21 days or mice were killed at indicated times and the lungs removed.

Transfer of in vitro generated T cells. Spleen cells from mice primed at least 3 weeks previously with A/PR/8/34 were restimulated in vitro with spleen cells infected with either A/PR/8/34, VV-PR8-HA6, VV-PR8-NP6, or VV-WR-TK⁻ at a responder-to-stimulator cell ratio of 5:1. After incubation for 5 days at 37°C, viable cells were separated on an Isopaque–Ficoll gradient. Five times 10^7 viable cells per mouse were inoculated i.v. into mice that had received i.n. 100 LD_{50} influenza virus 1 h earlier. The mice were monitored for mortality and morbidity or the lungs were removed at times indicated in the results.

Tc assay. Tc were generated in vitro and assayed on ^{51}Cr-labelled, virus-infected target cells over a range of effector to target ratios, as described in detail elsewhere [1].

Assay of lung influenza virus titres. The mice were killed by ether inhalation and the lungs removed and frozen in 1.0 ml phosphate-buffered saline (PBS) at −70°C. Before assay, the lungs were ground in a cold (−20°C) mortar and debris was removed by low speed centrifugation. Suitable 10-fold dilutions of supernatants were made in gelatin saline and 0.1 ml inoculated into the allantoic cavity of 10-day-old embryonated eggs (4–5 eggs were used for each dilution). After incubation for 48 h, the allantoic fluids were assayed for virus growth by haemagglutination and EID_{50}/lung calculated (Spearman-Karber method).

Assay of influenza-specific antibodies. Sera were assayed for haemagglutination-inhibiting (HAI) antibodies using chick erythrocytes as described [5]. Antibodies were also determined by ELISA assay. Influenza virus (100 HAU/well) in carbonate buffer was bound to polyvinyl immunoassay plates (Flow Laboratories, Uxbridge, Middlesex, UK) overnight at 4°C. After washing in PBS containing 1% (wt/vol) bovine serum albumin and 0.02% (wt/vol) NaN_3, serial dilutions of sera that had been treated with receptor-destroying enzyme and heat inactivated, were added to the plates. After incubation at room temperature for 90 min, the plates were washed and alkaline-phosphatase conjugated goat anti-mouse IgG (heavy+light) antibody (Cappel Worthington, West Chester, Pa., USA) was added, and the plates were kept at room temperature for a further 90 min. After washing, substrate (Sigma, St Louis, Mo. USA) was added and the reaction stopped at 30 min by the addition of 3M NaOH. Optical density in each well was read at 410 nm.

RESULTS

Protection of immunized mice

To assess the protective capacity of the vaccinia–influenza recombinants, groups of mice were immunized with either A/PR/8/34, VV-PR8-HA6, VV-PR8-NP6, or VV-WR-TK⁻, and 21 days later challenged i.n. with 100 LD_{50} A/PR/8/34. Fig. 1 shows survival rates over the succeeding 22 days. All mice immunized with either A/PR/8/34 or VV-PR8-HA6 survived the lethal challenge and had no signs of morbidity, i.e. no lethargy, hunching, weight loss, or rapid respiration. All mice immunized with either VV-PR8-NP6 or VV-WR-TK⁻ showed morbidity according to the above criteria. All but one of the 10 mice from the VV-WR-TK⁻-immunized group were dead by day 7. Six out of 10 of the VV-PR8-NP6-immunized group survived. Immunization with VV-PR8-NP6 did not protect mice against morbidity, but raised the survival rate from 10 to 60% when compared with mice immunized with VV-WR-TK⁻. Re-immunization of mice with 10^8 p.f.u.-VV-PR8-NP6 i.v. at day 21 and challenge 4 days later did not result in complete protection either (data not shown). In the two experiments performed with this re-immunization protocol, 80% of the VV-PR8-NP6-immunized mice survived, but still showed morbidity.

The survival rates of VV-PR8-NP6-immunized CBA/H mice varied between experiments. Overall, 24 out of 37 (64%) VV-PR8-NP6-immunized mice survived 10 LD_{50} A/PR/8/34 (six experiments with a range of 50–86% survival) and 18 out of 74 (24%) survived 100 LD_{50} A/PR/8/34 (10 experiments

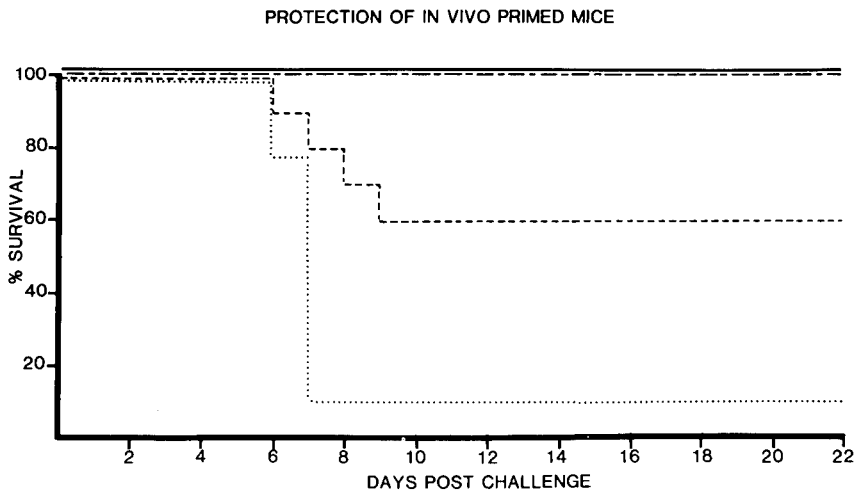

FIG. 1. Protection of in vivo-primed mice. Groups of 11 CBA/H female mice were immunized i.v. with the indicated virus, and after 21 days 10 mice per group were challenged i.n. with 100 LD_{50} A/PR/8/34. Splenocytes from the remaining mouse in each group were restimulated with A/PR/8/34 and assayed for Tc. The percentages in brackets represent the level of specific lysis of A/PR/8/34-infected target cells at an effector to target ratio of 5:1. The mice were primed with: —— A/PR/8/34 (49%), –·–· VV-PR8-HA6 (15%), – – – VV-PR8-NP6 (28%) and ···· VV-WR-TK$^-$ (6%).

with a range of 0–60% survival). In all experiments performed, A/PR/8/34 and VV-PR8-HA6 immunization totally protected all mice at challenge doses of 10, 100 or 1000 LD_{50} A/PR/8/34. Similar morbidity and mortality rates were observed in C57BL/6 and BALB/c strains of mice for VV-PR8-HA6 or VV-PR8-NP6 immunization (data not shown).

We have previously shown that both VV-PR8-HA6- and VV-PR8-NP6-primed Tc can be restimulated by A/PR/8/34 to recognize A/PR/8/34-infected target cells [1]. Splenocytes from one mouse from each group shown in Fig. 1 were re-stimulated in vitro with A/PR/8/34 and assayed for Tc. The level of lysis at an effector to target ratio of 5:1 was as follows: A/PR/8/34-immunized, 49%; VV-PR8-HA6-immunized, 15%; VV-PR8-NP6-immunized, 28%; and VV-WR-TK$^-$-immunized, 6%. The values indicate that VV-PR8-NP6 primed for a Tc response that was greater than that primed for by VV-PR8-HA6. Previous results have shown that the NP-specific Tc repertoire is approximately three-fold greater than the HA-specific Tc repertoire [1]. Despite priming for a substantial Tc response, VV-PR8-NP6 conferred poor protection when used to immunize mice, since all the mice became ill upon challenge with influenza virus and a variable percentage of mice survived.

Protection of mice by transferred T cells.

Transferred influenza-specific Tc can protect mice in terms of reduction in both mortality and lung virus titre [9, 17]. To test whether NP-specific T cells can protect mice against lethal influenza virus challenge, mice were challenged i.n. with 100 LD_{50} A/PR/8/34 and then inoculated i.v. with activated T cells generated in vitro by re-stimulation of A/PR/8/34-primed splenocytes with either A/PR/8/34, VV-PR8-HA6, VV-PR8-NP6, or VV-WR-TK$^-$. T cells specific for whole A/PR/8/34, HA, or NP were able to protect mice against mortality, whereas only one out of seven VV-WR-TK$^-$-re-stimulated T-cell recipient mice survived (Fig. 2). In addition, none of the mice that had received cells specific for whole A/PR/8/34, HA, or NP showed signs of morbidity, whereas all mice, including the surviving mouse, in the VV-WR-TK$^-$ group showed considerable morbidity. The data show that transferred NP-specific T cells offer complete protection.

To quantitate morbidity in both protection

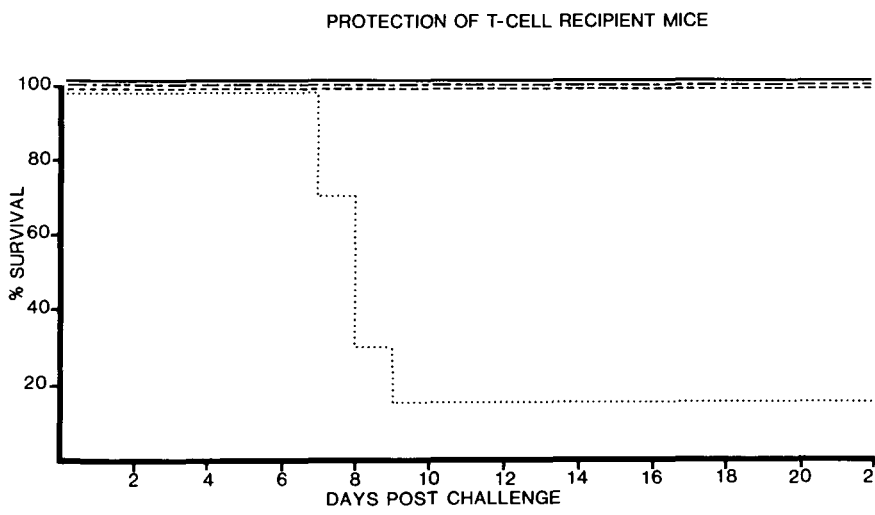

FIG. 2. Protection of T-cell recipient mice A/PR/8/34-primed CBA/H splenocytes were restimulated in vitro with A/PR/8/34, VV-PR8-HA6, VV-PR8-NP6, or VV-WR-TK$^-$. Groups of six to 10 mice were challenged i.n. with 100 LD_{50} A/PR/8/34 and 1 h later inoculated i.v. with 5×10^7 of the activated T cells. Specificity transferred cells: —— A/PR/8/34, –·–·– VV-PR8-HA6, – – – VV-PR8-NP6, ····· VV-WR-TK$^-$.

protocols, weight loss after lethal challenge of mice was assessed. Groups immunized with either VV-PR8-NP6 or VV-WR-TK$^-$ showed significant weight loss on day 6 after challenge when compared to groups immunized with either A/PR/8/34 or VV-PR8-HA6 (Table I, A). The smaller weight loss in VV-PR8-NP6-, compared with VV-WR-TK$^-$-immunized mice, was not a consistent finding. In T-cell recipient mice (Table I, B) only mice that received VV-WR-TK$^-$-restimulated T cells had a significant weight loss on day 6. Mice that received NP-specific T cells maintained the same weight as those that received A/PR/8/34- or HA-specific T cells. The difference in weights on day 0 between experiments A and B was accounted for by a difference in the gender of the mice used.

TABLE I. Weight loss in CBA/H mice challenged with 100 LD_{50} A/PR/8/34

	Weight in grams		Difference (day 6–day 0)
A. 21 days prior to challenge, male mice immunized with:	Day 0*	Day 6	
A/PR/8/34	26.7±1.5	29.0±1.5	2.3
VV-PR8-HA6	27.3±1.2	27.4±0.9	0.1
VV-PR8-NP6	27.3±1.5	23.8±2.1	−3.5
VV-WR-TK$^-$	25.8±1.2	19.3±1.3	−6.5
B. 1 h after challenge, female mice received cells re-stimulated with:			
A/PR/8/34	19.0±0.7	19.1±0.8	0.1
VV-PR8-HA6	18.9±1.1	18.9±0.7	0
VV-PR8-NP6	18.3±0.5	18.7±0.2	0.4
VV-WR-TK$^-$	18.9±0.7	14.0†	−4.9

* Day after intranasal challenge with 100 LD_{50} A/PR/8/34. Results are means of the weights of 4–8 mice±SD.

† Only one mouse survived to day 6. The result for five mice on day 4 was 14.4±0.8.

TABLE II. Lung virus titres in in vivo-immunized CBA/H mice

21 days prior to challenge, mice immunized with:	Log_{10} EID_{50} A/PR/8/34/Lung			Survival† (%)
	Day 2*	Day 4	Day 6	
A/PR/8/34	1.4±2.2‡§ (<1, <1, <1, <1 <1, <1.8, 4.7, 5.0)	<1	<1	100
VV-PR8-HA6	5.0±2.7‡ (<1, <1.7, 5.5, 5.9 5.9, 6.0, 6.9, 7.7)	<1	<1	100
VV-PR8-NP6	7.9±0.5	7.1±0.3**	5.2±0.5**	8
VV-WR-TK⁻	8.3±0.4	7.6±0.4	5.8±0.4	0

* Day after intranasal challenge with 100 LD_{50} A/PR/8/34. Values are means±SD of groups of eight mice; values for individual mice are given in brackets when there was a broad range of values.

† Groups of 13 mice monitored for survival for 21 days after challenge.

‡ ** Significantly different from VV-WR-TK⁻-immunized mice. ‡ ($P<0.005$) ** ($P<0.02$).

§ Significantly different from VV-PR8-HA6-immunized mice ($P<0.02$).

Reduction of lung virus titre by immunization or T-cell transfer

To elucidate the effect of VV-PR8-HA6 versus VV-PR8-NP6 immunization, influenza virus lung titres were assayed on days 2, 4 and 6 after challenge. Data from two experiments were pooled and are presented in Table II. A/PR/8/34 immunization substantially reduced the lung virus titre found on day 2; six mice had undetectable or barely detectable virus titres, and the remaining two mice had titres approximately 10^3-fold lower than VV-WR-TK⁻-immunized mice. VV-PR8-HA6 immunization did not reduce virus titres to the same extent; two mice had undetectable or barely detectable titres, and the remaining six mice had titres from 10- to 10^3-fold lower than control mice. Virus was undetectable by day 4 in the A/PR/8/34- and VV-PR8-HA6-immunized mice. In the pooled data in Table II, lung virus titres in the VV-PR8-NP6-immunized mice were slightly below control levels in VV-WR-TK⁻-immunized mice on days 4 or 6 ($P<0.02$). When the two experiments were analysed separately, the VV-PR8-NP6-immunized mice had lower titres on all days in both experiments, but these were significantly lower ($P<0.02$) in only one experiment. Lungs from both the VV-PR8-NP6- and VV-WR-TK⁻-immunized mice showed extensive consolidation on day 6 by macroscopic examination. Small areas of consolidation were seen in the lungs of A/PR/8/34- or VV-PR8-HA6-immunized mice.

Early reduction of lung virus titres in A/PR/8/34- and VV-PR8-HA6-immunized mice suggests that the mediator of protection in these groups is virus-neutralizing HA-specific antibody that reduces the initial rate of infection and rapidly clears virus when infection is established. Antibody levels on day 0 in the two groups of mice are shown in Table III. The VV-PR8-HA6-immunized mice had titres that were two-fold lower by HAI and four-fold lower by ELISA (to whole influenza virion). It is impossible to say unequivocally whether the difference in ability to prevent infection or to clear virus by day 2 can be accounted for by the difference in antibody titre.

When lung virus titres were assayed in mice that had received activated T cells, a different picture emerged (Table IV). On day 2 there was no significant reduction in lung virus titre in any group of mice that received cells. On day 4, the

TABLE III. Antibody levels in immunized CBA/H mice

21 days prior to challenge, mice immunized with:	Antibody titre at time of challenge	
	HAI titre	ELISA titre
A/PR/8/34	266* (160–320)	8,100*
VV-PR8-HA6	112 (80–160)	2,050 (900–2,700)

* Geometric means of antibody titres from groups of four mice, with the range of values in brackets.

TABLE IV. Lung virus titres in CBA/H T cell recipient mice

1 h after challenge mice received 5×10^7 cells re-stimulated with:	Log_{10} EID_{50} A/PR/8/34/lung			Survival† (%)	Lytic units transferred§
	Day 2*	Day 4	Day 6		
A/PR/8/34	7.8±0.3‡	5.1±0.4**	0.8±1.8** (<1, <1, <1, <1, 4.1)	100	1136
VV-PR8-HA6	8.5±0.3	5.6±0.4**	2.4±0.4**	100	178
VV-PR8-NP6	7.9±0.4	5.7±0.2**	1.5±1.5** (<1, <1, 1.75 2.3, 3.5)	100	1923
VV-WR-TK⁻	8.7±1.4	8.5±0.5	6.7±0.4	22	—
Nil cells	8.3±0.2	8.0±0.2	6.1±0.4	0	—

* As in Table II (three to five mice per group).
† Groups of four to nine mice monitored for survival for 21 days.
§ One lytic unit is defined as the number of T cells required to give 33% lysis on A/PR/8/34-infected target cells.
‡ ** Significantly different from mice that received no cells. ‡ ($P<0.005$), ** ($P<0.001$).

groups that received T cells specific for A/PR/8/34, HA, or NP had significantly reduced titres of lung virus; the titres were reduced further on day 6, survival was 100%, and there was no morbidity in these groups. Mice receiving VV-WR-TK⁻-restimulated cells had no reduction in lung virus titre when compared to mice that received no cells; however, two out of nine mice survived when monitored for 21 days. The data confirm that activated T cells act by promoting recovery from infection. T cells specific for whole A/PR/8/34, HA, or NP were equally protective at the dose given, despite a difference in the lytic capacity of the cells transferred.

DISCUSSION

We have shown that immunization of mice with VV-PR8-HA6, a vaccinia virus that expresses the HA from the influenza virus A/PR/8/34, totally protected mice from mortality and morbidity after a lethal challenge with A/PR/8/34 (Fig. 1, Table I). VV-PR8-HA6 immunization was as protective against mortality and morbidity as A/PR/8/34 immunization, even at the highest challenge dose tested, 1000 LD_{50}. When clearance of lung virus was examined, fewer VV-PR8-HA6-immunized mice had cleared the challenge virus by day 2 than A/PR/8/34-immunized mice, but all mice in both groups had cleared the virus by day 4 (Table II). Since VV-PR8-HA6 immunization of mice reduced lung virus titres early, the most significant mechanism of action was probably virus-neutralizing antibody, which reduced the initial rate of infection and rapidly cleared virus. The contention that antibody was the major mediator of protection is further supported by the fact that immunization of mice with another recombinant, VV-PR8-HA8, also confers total protection (data not shown). HA expression from VV-PR8-HA8 is regulated by a late vaccinia promoter, and we have reported that VV-PR8-HA8 does not prime Tc in CBA/H mice, but does stimulate a haemagglutination-inhibiting antibody response [5].

Immunization of mice with VV-PR8-NP6 conferred very little protection in terms of mortality (Fig. 1), morbidity (Table I), or reduction of lung virus titre (Table II). It is difficult to speculate on the mechanism responsible for survival in a small percentage of VV-PR8-NP6-immunized mice. The reductions in lung virus titres in Table II were only slightly below control levels in VV-WR-TK⁻-immunized mice ($P<0.02$), and this low level of significance was apparent in only one of the two experiments used for the data in Table II. Survival was also low in these two experiments. It is possible that a small decrease in lung virus titre can permit survival in a few mice.

When the recombinant viruses were used to restimulate T cells specific for either HA or NP, and those T cells transferred to recipient mice, both HA- and NP-specific T cells conferred protection, when assessed by morbidity and mortality rates, as did T cells restimulated by

A/PR/8/34 (Fig. 1, Table I). As would be expected [3, 18], the transferred T cells did not reduce the initial rate of infection, but started to clear virus by day 4 and further reduced virus titres by day 6 (Table IV). It is not know whether there would be a difference in protective capacity of the different T-cell populations at lower numbers of transferred cells.

We [1] and others [19] have shown that vaccinia-NP recombinant viruses prime for Tc in mice. It is not clear why these Tc do not offer better protection to VV-PR8-NP6-immunized mice when they are challenged with A/PR/8/34. Influenza-specific Tc have been demonstrated in mouse lung after immunization with influenza [18] or a vaccinia-NP recombinant virus [19]. Transferred T cells may function for one of two reasons: (i) in vitro-stimulated T cells usually traffic to and persist in lungs [10], or (ii) the transferred T cells are in an activated state, whereas the in vivo-stimulated T cells had quiesced to a memory state when the mice were challenged on day 21. VV-PR8-NP6-immunized mice that were re-immunized with VV-PR8-NP6 on day 21 and challenged 4 days later were still not totally protected, which favours the first possibility (data not shown). However, although re-immunization with vaccinia virus recombinants boosts the antibody response to the foreign antigen, it does not result in detectable Tc in spleen (unpublished observations).

As influenza virus changes antigenically over time and it is not yet possible to predict the antigenicity of viruses responsible for future pandemics, a vaccine that promotes cross-reactive immunity would be highly advantageous. Influenza virus infection in humans confers little protection against subsequent infection with a different subtype virus, showing that natural infection does not stimulate a strong cross-reactive protective response. It may be possible to manipulate the immune response to generate a cross-protective response by vaccinating with a common antigen such as matrix protein [8] or NP; however, the data presented here show that immunization with NP alone is not sufficient to confer good protection, despite the ability of transferred NP-specific T cells to accelerate the clearance of influenza virus from lungs and thus protect all mice against morbidity and mortality. The data also show that immunization with influenza HA alone is sufficient to confer total protection from both mortality and morbidity in mice challenged with a lethal dose of homologous influenza virus, and demonstrate the efficacy of delivering a single viral antigen via a live vector such as vaccinia virus.

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